which N-terminal deletions had been made to create portions of agrin (designated delta 3 through 9) as follows:

delta 3: agrin sequence starts with amino acid #60: QTAS...(SEQ ID NO: 25)
delta 4: agrin sequence starts with amino acid #76: NGFS...(SEQ ID NO: 26)
delta 5: agrin sequence starts with amino acid #126: VSLA...(SEQ ID NO: 27)
delta 6: agrin sequence starts with amino acid #178: GPRV...(SEQ ID NO: 28)
delta 7: agrin sequence starts with amino acid #222: GFDG...(SEQ ID NO: 29)
delta 8: agrin sequence starts with amino acid #260: ASGH...(SEQ ID NO: 30)
delta 9: agrin sequence starts with amino acid #300: AGDV...(SEQ ID NO: 31)

All of the sequences continue to the terminal amino acids PCPTP, as with the 50kD agrin.

REMARKS

This Preliminary Amendment is made merely to insert the priority data; to cancel claims 1-11, 25-37, 48; 55, 58, and 62.to correctly label Figure 1 as Figure 1A - 1D, Figure 4 as Figure 4A-4D, Figure 14 as Figure 14A-14C, and Figure 15 as Figure 15A-15B;; and to add the sequence identifiers to the specification.

Applicants submit herewith as Exhibit A: Marked-Up Versions of pages 1, 11, 12, 13, 18, 19, 20, 21, 24, 36, 37, 38, 50, 51, 57, 64, 75, 76, 77, 78, 79, 83, 89, 92, 93, and 94; Exhibit B: Sequence Listing in paper form.

The computer readable form of the "Sequence Listing" in this application, filed herewith, is identical with that filed in USSN 09/077,955, filed September 10, 1998. In accordance with 37 C.F.R. § 1.821(e), please use the first-filed computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable

form that will be used for the instant application. A paper copy of the Sequence Listings is included as Exhibit B.

I hereby state that the content of the paper readable and computer readable copy of the Sequence Listing submitted herewith and referred to herein in accordance with 37 C.F.R. § 1.821(g), contain no new subject matter.

Applicants direct the subject Sequence Listings submitted herewith be added to the specification.

No fee is deemed necessary for filing this paper. However, if any fees are deemed necessary, the Commissioner is hereby authorized to charge any such fees required by this paper to Deposit Account No. 18-0650.

Respectfully submitted,

Linda O. Palladino

Gail M. Kempler
Reg. No. 32,143
Joseph M. Sorrentino
Reg. No. 32,598
Attorneys for Applicants
Linda O. Palladino
Reg. No. 45,636
Patent Agent for Applicants
Regeneron Pharmaceuticals, Inc.
777 Old Saw Mill River Road
Tarrytown, New York 10591
(914) 345-7400

NOVEL TYROSINE KINASE RECEPTORS AND LIGANDS

This application is a divisional application of United States Application No. 09/077,955 filed September 10, 1998, which ~~This application~~ claims priority of United States Application Serial No. 08/644,271 filed May 10, 1996 and of United States Provisional Application No. 60/008,657 filed December 15, 1995, each of which is incorporated by reference herein.

Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

INTRODUCTION

The present invention provides for a novel receptor molecule, a novel molecule capable of activating the receptor, and methods of making and use thereof.

BACKGROUND OF THE INVENTION

The ability of polypeptide ligands to bind cells and thereby elicit a phenotypic response such as cell growth, survival or differentiation in such cells is often mediated through receptor tyrosine kinases. The extracellular portion of each receptor tyrosine kinase (RTK) is generally the most distinctive portion of the molecule, as it provides the protein with its ligand-recognizing characteristic. Binding of a ligand to the extracellular domain results in signal transduction via an intracellular tyrosine kinase catalytic domain which transmits a biological signal to intracellular target proteins. The particular array of sequence motifs of this cytoplasmic, catalytic domain determines its access to potential kinase substrates (Mohammadi, et al., 1990, Mol. Cell. Biol., 11: 5068-5078; Fantl, et al., 1992, Cell, 69:413-413).

MARKED-UP VERSION

The invention further provides for an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence comprising the coding region of the active portion of human agrin contained in the vector designated as pBL-hAgrin 1 (ATCC Accession No. 97378);

(b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a) and which encodes the active portion of human agrin; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes the active portion of human agrin.

The invention also provides for the above-described nucleic acid molecule which additionally contains a nucleotide sequence so that the encoded polypeptide contains the eight amino acids ELANEIPV at the position corresponding to amino acid position 1780 as shown in Figure 14~~A~~-14C (SEQ ID NO:34).

The invention also provides for a method of promoting the growth, survival or differentiation of a MuSK receptor expressing cell comprising administering to the MuSK receptor expressing cell an effective amount of agrin or a derivative of agrin. The method may be practiced in vitro or in vivo. In one embodiment of this method, the agrin is human agrin. In another embodiment of this method, the MuSK receptor expressing cell is a cell which is normally found in the heart, spleen, ovary, retina or skeletal muscle. In another embodiment, the MuSK receptor expressing cell is a cell which has been genetically engineered to express the MuSK receptor.

The present invention also includes a method of treating a patient suffering from a muscle disease or neuromuscular disorder comprising administering

to the patient an effective amount of agrin or a derivative thereof. By way of non-limiting example, the agrin may be human agrin and the derivative may be the active portion of the human agrin molecule.

The present invention also includes an antibody capable of specifically binding human agrin. More specifically, the invention includes an antibody capable of specifically binding the active portion of human agrin. The antibody may be monoclonal or polyclonal. The invention further provides a method of detecting the presence of human agrin in a sample comprising:

a) reacting the sample with an antibody capable of specifically binding human agrin under conditions whereby the antibody binds to human agrin present in the sample; and

b) detecting the bound antibody, thereby detecting the presence of human agrin in the sample.

The antibody used may be monoclonal or polyclonal. The sample may be biological tissue or body fluid. The biological tissue may be brain, muscle, or spinal cord. The body fluid may be cerebrospinal fluid, urine, saliva, blood, or a blood fraction such as serum or plasma.

The invention further provides for an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding human muscle specific kinase (MuSK) receptor, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence comprising the coding region of the human MuSK receptor as set forth in Figure 4A-4D (SEQ ID NO: 33)

(b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a) and which encodes a human MuSK receptor; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes a human MuSK receptor.

BRIEF DESCRIPTION OF THE FIGURES

A-10 (SEQ ID NO: 2)

FIGURE 1 - Nucleotide and deduced amino acid (single letter code) (SEQ ID NO: 1) sequences of rat musk. The nucleotide sequence encoding mature MuSK begins around nucleotide 192.

FIGURE 2 - Northern blot showing distribution of musk in the rat during early development. Lane 1: Total embryo E9; Lane 2: Total embryo E11; Lane 3: Placenta E11; Lane 4: Embryo head E12; Lane 5: Embryo body E12; Lane 6: Embryo spinal cord E12; Lane 7: Placenta E12; Lane 8: Embryo head E13; Lane 9: Embryo body E13; Lane 10: Embryo brain E17; Lane 11: Embryo brain P1; Lane 12: Embryo brain P10; Lane 13: Embryo brain P19; Lane 14: Adult brain; Lane 15: Adult muscle; Lane 16: Adult denervated muscle; where day of sperm positivity is designated as day E1, and day of birth is designated as day P1.

FIGURE 3 - Northern blot showing distribution of musk in adult rat tissues. Lane 1: Brain; Lane 2: Olfactory bulb; Lane 3: Cortex; Lane 4: Hippocampus; Lane 5: Thalamus/hypothalamus; Lane 6: Midbrain; Lane 7: Hindbrain; Lane 8: Cerebellum; Lane 9: Spinal Cord; Lane 10: Thymus; Lane 11: Spleen; Lane 12: Liver; Lane 13: Kidney; Lane 14: Lung; Lane 15: Sciatic Nerve; Lane 16: Retina; Lane 17: Heart; Lane 18: Ovary ; Lane 19: Muscle; Lane 20: Denervated muscle.

A-40 (SEQ ID NO: 32)

FIGURE 4 - Nucleotide and deduced amino acid (single letter code) (SEQ ID NO: 33) sequences of human MuSK receptor.

complexes on the cell surface with agrin and the myotube-specific accessory component.

A-14C (SEQ ID NO: 34)
FIGURE 14 - Amino acid (single letter code) sequence of rat agrin indicating Y and Z sites of amino acid inserts found in splice variants.

A-15B (SEQ ID NO: 35) (SEQ ID NO: 36)
FIGURE 15 - Nucleotide and amino acid (single letter code) sequences of human agrin expression construct including the signal peptide and flg tag (FLAG tag). The start of the coding region for the active C-terminal fragment (portion) of human agrin 4-8 is indicated. Also indicated are the position Y and position Z insert sites at which the 4 and 8 amino acid inserts are located. Throughout this application, references to human agrin 4,8; c-agrin 4,8; or human c-agrin 4,8 indicate the active C-terminal fragment (portion) of human agrin 4-8 as set forth in the Figure.

FIGURE 16 - Results of phosphorylation assay showing that the active C-terminal 50kD portion of human agrin 4,8 and the truncated delta 9 portion of human agrin can each induce phosphorylation of the MuSK receptor.

FIGURE 17 - Results of pharmacokinetic study comparing serum half-lives of active C-terminal 50kD portion of human agrin 4,8 (c-agrin 4,8) with active C-terminal 50kD portion of human agrin 4,8 that has been modified by covalent addition of polyethylene glycol.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides for a novel tyrosine kinase molecule that is related to the trk family of tyrosine kinases. The sequence of the protein is set forth in Figure 1 as SEQ. ID NO: 1. The coding region of the mature protein is

believed to begin on or around the serine-glycine-threonine on or around position 20 of the coded region.

The novel tyrosine kinase described herein has been found to be induced in denervated skeletal muscle. Accordingly, it has been designated as MuSK (muscle specific kinase). It has also been referred to previously as Dmk (denervated muscle kinase). In addition to being found in skeletal muscle, both normal and denervated, MuSK has also been found to be present in, but not be limited to, the spleen, ovary and retina. It appears to be present during early development, but is also found in adult tissue.

MuSK may be related to the Torpedo RTK identified by Jennings, et al. supra. However, MuSK differs in that it appears to be induced in denervated muscle, whereas no such induction has been reported with regard to the Torpedo RTK. Furthermore, the Torpedo RTK has an extracellular kringle domain, whereas MuSK does not. However, these kinases may be members of the same or related families.

The gene encoding rat MuSK has been cloned and the DNA sequence determined (Figure 1A-1D; SEQ ID NO: 2). The extracellular domain of the mature protein is believed to be encoded by the nucleotide sequence beginning on or around position 192 and ending on or around position 1610. The transmembrane portion of the protein is believed to be encoded by the nucleotide sequence beginning on or around position 1611 and ending on or around position 1697. The intracellular domain is believed to be encoded by the nucleotide sequence beginning on or around position 1698 and ending on or around position 2738. A cDNA clone encoding Dmk (MuSK) was deposited with the American Type Culture Collection on July 13, 1993 and accorded an accession number of ATCC No. 75498.

The present invention also provides for a protein or peptide that comprises the extracellular domain of MuSK as well as the sequence of nucleotides which encode this extracellular domain. The extracellular domain of the protein is believed to be comprised of the amino acids at or around positions 20 through 492 of the coding region set forth as SEQ ID NO: 1.

The similarity between MuSK and the Torpedo RTK suggests the utilization of regions of sequence homologies within these genes to develop primers useful for searching for additional, related RTKs.

Accordingly, the invention provides for nucleic acids, or oligonucleotides greater than about 10 bases in length, that hybridize to the nucleic acid sequences described herein and that remain stably bound under stringent conditions. Stringent conditions as used herein are those which (1) employ low ionic strength and high temperature for washing, for example, 0.15 M NaCl/ 0.015 M sodium citrate /0.1% $NaDodSO_4$ at 50°C, or (2) use during hybridization of a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C.

The present invention further provides for an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding human muscle specific kinase (MuSK) receptor, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence comprising the coding region of the human MuSK receptor as set forth in Figure 4A-4D (SEQ ID NO. 30);

(b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a) and which encodes a human MuSK receptor; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes a human MuSK receptor.

The invention further provides for isolated and purified human MuSK receptor encoded by the coding region of the human MuSK receptor nucleotide sequence as set forth above. The invention also provides for a vector which comprises the isolated nucleic acid molecule described. In one embodiment, the vector is an expression vector wherein the DNA molecule is operatively linked to an expression control sequence. In a further embodiment, the expression vector comprises an immediate early gene promoter. In a still further embodiment, the expression vector of the invention comprises the fos promoter or the jun promoter as the early gene promoter.

The invention further contemplates a host-vector system for the production of a polypeptide having the biological activity of a human MuSK receptor which comprises the vector described above in a suitable host cell. By way of nonlimiting example, a suitable host cell may be a C2C12 cell or an NIH3T3 cell. The invention further provides for a method of producing a polypeptide having the biological activity of human MuSK receptor which comprises growing cells of the above-described host-vector system under conditions permitting production of the polypeptide and recovering the polypeptide so produced.

In addition, the invention provides for a therapeutic composition comprising the MuSK receptor activating molecule in a pharmaceutically acceptable vehicle.

MARKED-UP VERSION

3'

1) Asp-Val-Trp-Ser-Leu-Gly (SEQ ID NO: 9)
3'-CTRCANACCWSNATRCCCTCGAGCTTAAG-5' (SEQ ID NO: 10)

2) Asp-Val-Trp-Ser-Phe-Gly (SEQ ID NO: 11)
3'-CTRCANACCWSNAARCCCTCGAGCTTAAG-5' (SEQ ID NO: 12)

3) Asp-Val-Trp-Ser-Tyr-Gly (SEQ ID NO: 13)
3'-CTRCANACCWSNRANCCCTCGAGCTTAAG-5' (SEQ ID NO:14)

Alternatively, regions of homology shared by MuSK and members of related families, such as the Trk family, may be used in strategies designed to isolate novel RTKs.

The present invention further provides for substantially purified protein molecules comprising the amino acid sequence substantially as set forth in Figure 1A-1D for MuSK (SEQ ID NO: 1) or functionally equivalent molecules. Functionally equivalent molecules include derivatives in which amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the

The invention further provides for an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence comprising the coding region of the active portion of human agrin contained in the vector designated as pBL-hAgrin 1 (ATCC Accession No. 97378);

(b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a) and which encodes the active portion of human agrin; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes the active portion of human agrin.

The invention also provides for the above-described nucleic acid molecule which additionally contains a nucleotide sequence so that the encoded polypeptide contains the eight amino acids ELANEIPV at the position corresponding to amino acid position 1780 as shown in Figure 14. A-14C (SEQ ID NO: 34)

The invention further provides for an isolated and purified nucleic acid molecule comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence comprising the coding region of the active portion of human agrin as set forth in Figure 15; A-15B (SEQ ID NO: 35)

(b) a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of (a) and which encodes the active portion of human agrin; and

(c) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a) or (b) and which encodes the active portion of human agrin.

The invention further provides for an isolated nucleic acid molecule comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is selected from the group consisting of:

(a) the nucleotide sequence as set forth in Figure 15A-15B (SEQ ID NO: 35);

(b) the nucleotide sequence encoding amino acids 24 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(c) the nucleotide sequence encoding amino acids 60 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(d) the nucleotide sequence encoding amino acids 76 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(e) the nucleotide sequence encoding amino acids 126 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(f) the nucleotide sequence encoding amino acids 178 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(g) the nucleotide sequence encoding amino acids 222 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(h) the nucleotide sequence encoding amino acids 260 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(i) the nucleotide sequence encoding amino acids 300 to 492 as set forth in Figure 15A-15B (SEQ ID NO: 35);

(j) a nucleotide sequence that hybridizes under stringent conditions to any of the nucleotide sequences of (a) through (i) and which encodes the active portion of human agrin; and

(k) a nucleotide sequence that, as a result of the degeneracy of the genetic code, differs from any of the nucleotide sequences of (a) through (j) and which encodes the active portion of human agrin.

A further embodiment of the invention is an isolated and purified nucleic acid molecule encoding agrin 0-8 comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is as set

A-15B (SEQ ID NO: 35)
forth in Figure 15 with the exception that there is no insert at position Y. Another embodiment of the invention is an isolated and purified nucleic acid molecule encoding agrin 4-0 comprising a nucleotide sequence encoding the active portion of human agrin, wherein the nucleotide sequence is as set forth
A-15B (SEQ ID NO: 35)
in Figure 15 with the exception that there is no insert at position Z.

The present invention provides for an isolated polypeptide encoded by any one of the nucleic acid molecules of the invention as set forth herein. Furthermore, the present invention provides for said polypeptides modified by covalent attachment of a polyethylene glycol molecule.

Thus, the present invention provides truncated forms of the human agrin polypeptide which retain one or more of the biological activities of human agrin. As set forth herein, the invention also provides nucleic acid sequences encoding such truncated forms. These truncated forms retain, for example, the ability to induce phosphorylation of the MuSK receptor. The truncated forms may be of any suitable length, as long as they retain one or more of the biological activities of human agrin. Truncated forms including the C-terminal of human agrin are preferred.

A-15B (SEQ ID NO: 35)
Referring to Figure 15, starting at the N-terminal end (amino acid 24 - KSPC) these truncated forms of human agrin preferably have deletions of up to 10, 20, 30, 40, 50, 100, 150, 200, 250, 300, 350 or 400 amino acids. Particularly preferred truncated forms are described herein as delta 3 through delta 9.

The invention also provides for a method of promoting the growth or survival of a MuSK receptor expressing cell in culture comprising administering to the MuSK receptor expressing cell an effective amount of agrin or a derivative of agrin. In one embodiment of this method, the agrin is human agrin. In another embodiment of this method, the MuSK receptor expressing cell is a cell which is normally found in the heart, spleen, ovary or

5'-TCTTGACTCGAGAYYTNGCNGCNMGNAA-3' (SEQ ID NO: 8)
5'-GAATTCGAGCTCCCRTANSWCCANACRTC-3' (SEQ ID NO: 15)
with which to prime PCR reactions using denervated muscle cDNAs. Resulting amplified DNA fragments were cloned by insertion into plasmids, sequenced and the DNA sequences were compared with those of all known tyrosine kinases. cDNA templates were generated by reverse transcription of denervated muscle tissue RNAs using oligo d(T) primers. PCR reactions were done at primer annealing temperatures of 40°C. Aliquots of the PCR reactions were subjected to electrophoresis on an agarose gel.

Size-selected amplified DNA fragments from these PCR reactions were cloned into plasmids as follows: Each PCR reaction was reamplified as described above, digested with XhoI and SacI to cleave sites in the termini of the primers (see below). XhoI/SacI-cut DNAs were purified by Magic PCR kit (from Promega) and cloned into compatible XhoI/SacI sites in the Bluescript II SK(+) plasmid, introduced into DH10B E. coli by electroporation, followed by plating of transformants on selective agar. Ampicillin-resistant bacterial colonies from PCR transformation were inoculated into 96-well microtiter plates and used for PCR using vector primers (T3 and T7) flanking the tyrosine kinase insert and these PCR fragments were analyzed by sequencing.

One of the cloned fragment sequences contained a segment of a novel tyrosine kinase domain, which was designated as MuSK. The sequence of the PCR-derived fragment corresponding to MuSK was used to generate PCR primers to obtain longer MuSK specific fragments by the RACE procedure. These longer MuSK probes were used as a hybridization probe to obtain full length MuSK cDNA clones from a rat denervated skeletal muscle cDNA library. DNA was sequenced by using the ABI 373A DNA sequencer and Taq Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of MuSK (Figure 1; SEQ ID NO:1) has a high degree of

A-10 E

homology to members of the trk family of proteins. It was also found to be similar to the Jennings, et al. Torpedo RTK found in muscle.

Oligonucleotide primers corresponding to conserved regions of known tyrosine kinase molecules were used to amplify and clone DNA sequences encoding novel orphan tyrosine kinase receptor molecules. The amino acid sequences of representatives from branches of the tyrosine kinase family and regions of homology within the catalytic domain of these proteins were used to design degenerate oligonucleotide primers. These primers were then used to prime PCR reactions using as template a rat denervated muscle cDNA library. Resulting amplified DNA fragments were then cloned into Bluescript II SK(+) plasmid, sequenced, and the DNA sequences compared with those of known tyrosine kinases. The sequence of a PCR fragment encoding a novel tyrosine kinase named MuSK was used to obtain more adjoining DNA sequence. A DNA fragment containing MuSK sequences was used as a probe to obtain a cDNA clone from a denervated skeletal muscle library. This clone encodes a novel tyrosine kinase receptor with a high degree of homology to members of the trk family of proteins. It was also found to be homologous to the Jennings, et al. Torpedo RTK. Figure 1A-1D presents the nucleotide sequence (SEQ ID NO: 2) of the musk clone.

EXAMPLE 2 - IDENTIFICATION OF ADDITIONAL TYROSINE KINASES

The novel MuSK sequence is used to obtain homology segments among receptor tyrosine kinases which can be used in combination with other homology segments. For example, an alignment of the Torpedo trk-related kinase with MuSK shows the following conserved protein segment:

Asp-Val-Trp-Ala-Tyr-Gly (SEQ ID NO: 3)

This homology "box" is not present in any other mammalian tyrosine kinase receptor. Degenerated oligonucleotides essentially based on this "box" in

MARKED-UP VERSION

EXAMPLE 5 - SEQUENCING OF HUMAN MuSK RECEPTOR

In order to obtain the full coding sequence of the human MuSK receptor, oligonucleotides based on the rat sequence were utilized as PCR primers to amplify cDNA from a human muscle biopsy. The PCR fragment so produced was then sequenced and the resulting new sequence corresponded to a partial sequence of the human MuSK receptor. The novel partial human MuSK receptor sequence was then used to obtain further sequence through successive rounds of the RACE procedure. (Frohman, M. A. (1990), RACE: Rapid amplification of cDNA ends. in PCR Protocols, Innis, M.A.Gelfand, D.H., Snincky, J.J., and White, T.J. eds. Academic Press. San Diego).

This process was complemented by obtaining human genomic clones of MuSK and using the coding sequence of the genomic MuSK to design oligonuclotide primers used to amplify the biopsy cDNA. Stretches of the human MuSK cDNA sequence which were difficult to sequence, absent or presenting some ambiguity were confirmed, corrected or added from the human genomic MuSK sequence. MuSK cDNA variants produced by alternative splicing of MuSK transcripts may be obtained by using this sequence to obtain MuSK cDNA from human sources. The deduced amino acid sequence of the human MuSK receptor and the nucleotide sequence encoding it is set forth in Figure 4. One of skill in the art will readily recognize that this sequence may be used to clone full length, naturally occurring cDNA sequences encoding the human MuSK receptor, which may vary slightly from the sequence set forth in Figure 4? A-4D (SEQ ID NO: 32)

these possibilities, applicants took advantage of the fact that RTKs become rapidly autophosphorylated on tyrosine upon challenge with their cognate ligand. Applicants decided to assay four of the known forms of soluble agrin - which exhibit differing AChR clustering activities (Ruegg, M.A. et al., 1992, Neuron 8: 691-699; Ferns, M., et al., 1992, Neuron 8: 1079-1086; Ferns, M., et al., 1993, Neuron 11: 491-502; Hoch, W. et al., 1994, EMBO J. 13: 2814-2821) - for their ability to induce phosphorylation of the MuSK receptor.

The ability of various agrins and growth factors to induce MuSK or ErbB3 tyrosine phosphorylation, for the indicated times and at the indicated concentrations, was evaluated in primary rat myoblasts and in either untransfected C2C12 myoblasts, or in C2C12 myoblasts stably transfected with a chick MuSK-expressing plasmid. The cells were challenged at confluence in an undifferentiated state, or approximately 4-5 days after being induced to differentiate into myotubes in serum-poor media. After challenge, the cells were lysed, the extracts subjected to immunoprecipitation with receptor-specific antibodies, and then immunoblotted with either receptor-specific or phosphotyrosine-specific antibodies, using methods previously described (Stitt, T., et al., 1995, Cell 80: 661-670). Polyclonal antibodies for MuSK were generated as follows: for rat MuSK, rabbits were immunized with a peptide corresponding to the carboxy-terminal 20 amino acids of the rat MuSK protein (Valenzuela, D., et al., 1995, Neuron 15: 573-584; the nomenclature for this antibody is: 41101K); for chick MuSK, rabbits were immunized with a peptide corresponding to the first 19 amino acids of the chick MuSK cytoplasmic domain (Peptide: TLPSELLLDRLHPNPMYQ; (SEQ ID NO:16) the nomenclature for this antibody is 52307K). The specificity of the antibodies was determined on Cos-cell expressed MuSK proteins, by both immune-precipitation and Western, comparing untransfected Cos cell lysates to lysates from rat and chicken-MuSK transfected Cos cells. 41101K immune precipitates and Westerns rodent MuSK, but does not recognize chicken

Two pairs of PCR primers were synthesized based on human agrin cDNA sequences obtained from Genbank. The sequences of the oligonucleotide primers were as follows:

Primer pair 18:
h-agrin 18-5' : 5'-GACGACCTCTTCCGGAATTC-3' (SEQ ID NO: 17)
h-agrin 18-3' : 5'-GTGCACATCCACAATGGC-3' (SEQ ID NO: 18)

Primer pair 35:
h-agrin 35-5' : 5'-GAGCAGAGGGAAGGTTCCCTG-3' (SEQ ID NO: 19)
h-agrin 35-3' : 5'-TCATTGTCCCAGCTGCGTGG-3' (SEQ ID NO: 20)

The oligonucleotide primers were used for PCR amplification of two segments of DNA of approximately 100 nts (primer pair 18) and 85 nts (primer pair 35) using 300 ngs of human genomic DNA as a template. The PCR amplification was carried out as recommended by the manufacturer (Perkin-Elmer) under the following conditions: 35 cycles at 94°C for 60 sec, 55°C for 50 sec and 72°C for 30 sec. The PCR fragments obtained were purified from an agarose gel and re-amplified for 30 cycles using the same PCR conditions described above.

After amplification, the PCR reactions were electrophoresed in agarose gels, the agarose containing the DNA bands of 100 and 85 nts respectively was excised, purified by QiaEx II (Qiagen), and then cloned into plasmid pCR-script using Stratagene's pCR-Script cloning kit, followed by bacterial transformation and plating onto agar-ampicillin plates as recommended by the manufacturer. Bacterial colonies containing the 100 and 85 nt inserts were identified by PCR using the primers described above. The PCR fragments obtained were radiolabeled for use as probes using a standard PCR reaction (Perkin-Elmer) on 20 ng of DNA template, except that 5 nmoles each of dATP, dGTP and dTTP and 0.2 mCurie of alpha ^{32}P-dCTP (Du Pont 3000 Ci/mmol)

were added to the reaction mixture and then subjected to 7 cycles of PCR. Unincorporated label was separated from the probes on a G50 NICK column (Pharmacia). These probes were used to screen a human fetal brain cDNA library (Stratagene Cat# 936206) using standard library screening procedures (Sambrook, Fritsch and Maniatis, Molecular Cloning, a Laboratory Manual, (1989) Second Edition, Cold Spring Harbor Laboratory Press). One and a half million phage plaques were plated in XL-1 Blue bacteria as recommended by Stratagene, and transferred to nitrocellulose filters in duplicate as previously described (Id.). The filters were processed and each filter replica was used for hybridization with one of the above probes as previously described (Id.). Plaques hybridizing to both probes were isolated and purified and a plasmid containing the cDNA insert was excised from the lambda clone according to Stratagene's recommended procedure (EXASSIST/SOLR System). The pBluescript plasmid containing the human Agrin insert was purified and the insert was then sequenced using an automated sequencing kit (Applied Biosystems).

As a result of this screen, one clone (pBL-hAgrin1) was obtained which contains a nucleotide sequence encoding an amino acid sequence of human agrin. The first amino acid encoded by the cloned nucleotide sequence corresponds approximately to amino acid 424 of rat agrin (See Figure 14A-14C). The nucleotide sequence of the clone ends downstream of the stop codon. Clone pBL-hAgrin1 contains a 4 amino acid insert starting at the position which corresponds to position 1643 of Figure 14A-14C, a point which was previously described for the rat as position "Y" (Stone, D.M. and Nikolics, K., J. Neurosci. 15: 6767-6778 (1995)). The sequence of the 4 amino acid insert both in clone pBL-hAgrin1 and in the rat is KSRK.

A second clone was obtained from this screen. This second clone (pBL-hAgrin23) also contains a nucleotide sequence encoding an amino acid sequence of human agrin. The first amino acid encoded by the cloned

nucleotide sequence corresponds approximately to amino acid 1552 of the rat agrin (See Figure 14A-14C [SEQ ID NO: 34]). The nucleotide sequence of the clone ends downstream of the stop codon. Clone pBL-hAgrin23 contains an 8 amino acid insert starting at a position which corresponds to position 1780 of Figure 14A-14C (SEQ ID NO: 34), a point which was previously described for the rat as position "Z" (Stone, D.M. and Nikolics, K., J. Neurosci. 15: 6767-6778 (1995)). The sequence of the eight amino acid insert both in clone pBL-hAgrin23 and in the rat is ELANEIPV. As previously discussed, it has been reported that the 8 amino acid insert plays an important role in regulating the AChR clustering activity of different agrin forms. Therefore, by inserting a nucleotide sequence encoding the eight amino acid sequence ELANEIPV into clone pBL-hAgrin1 at the position corresponding to position Z of rat agrin, a human 4-8 agrin clone may be obtained. The addition of the 8 amino acid insert at position Z should confer a high level of biological activity to the human 4-8 clone.

Clone pBL-hAgrin23 also contains the 4 amino acid "Y" insert as described above for clone pBL-hAgrin1. However, clone pBL-hAgrin23 contains 17 extra amino acids at the same "Y" position, such that the sequence of the "Y" insert in clone pBL-hAgrin23 is KSRKVLSASHPLTVSGASTPR. Therefore, in addition to the (4-0) and (4-8) human agrin splice variants described above, human clones corresponding to splice variants containing (Y-Z) inserts of (17-0), (17-8), (21-0), and (21-8) are indicated by these results and are within the scope of the present invention.

EXAMPLE 15 - EXPRESSION OF HUMAN AGRIN

Construction of human agrin expression vector

A human agrin Sfi I - Aat II fragment containing the 4 amino acid insert at the position corresponding to the Y-site described for rat agrin (see Figure 14A-14C (SEQ ID NO: 34))

was excised from clone pBL h agrin-1. A human agrin Aat II - Not I fragment containing the 8 amino acid insert at the position corresponding to the Z-site described for rat agrin (see Figure 14A-14C [SEQ ID NO: 34]) was excised from clone pBL h agrin-23. A Xho I - Sfi I fragment was then generated via PCR that contained a preprotrypsin signal peptide, the 8 amino acid flg peptide (from the flag tagging system, IBI/Kodak, Rochester, NY) and the human agrin sequence corresponding to the sequence of amino acids from position 1480 to the Sfi I site located at amino acids 1563-1566 of rat agrin (see Figure 14A-14C [SEQ ID NO: 34]). The three fragments were then ligated into a Xho I - Not I digested pMT21 expression vector to form the human agrin 4-8 expression vector pMT21-agrin 4-8. The sequence of human agrin 4-8 that was encoded in the expression vector is shown in Figure 15A-B (SEQ ID NO: 35). Expression vectors for the human clones corresponding to splice variants containing (Y-Z) inserts of (0-8) and (4-0) were also constructed.

Expression of human agrin (4-8) in E. coli

The gene for human agrin 4-8 was PCR amplified from pMT21-agrin 4-8 with the primer pair AG5' (5'-GAGAGAGGTTTAAACATGAGCCCCTGCCAGCCCAACCCCTG-3' [SEQ ID NO: 21]) and AG3' (5'-CTCTGCGGCCGCTTATCATGGGGTGGGGCAGGGCCGCAG-3' [SEQ ID NO: 22]). The PCR product was digested with the restriction enzymes Pme I and Not I and cloned into the Pme I and Not I sites of the vector pRG501, a pMB1 replicon that confers kanamycin resistance and is designed to express cloned genes from the phage T7 promoter. One isolate was characterized and named pRG531. The 1315 base pair Nco I - Nae I fragment internal to agrin in pRG531 was then replaced with the corresponding fragment from pMT21-agrin 4-8. The resulting plasmid, pRG451, was transformed into the expression strain RFJ209 [IN(rrnD-rrn/E)1 $lacI^Q$ lacZpL8 fhuAD322-405 $rpoS_{(MC4100)}$ ara::(lacUV5-T7 gene 1)8]. Cultures of RFJ209 / pRG541 induced with IPTG express human agrin to about 5% of total cellular protein and

fractionates with soluble protein upon cell disruption. The crude soluble protein fraction containing human agrin 4-8, as well as human agrin 4-8 purified by Q-Sepharose chromatography was determined to be active in phosphorylation of MuSK receptor.

Expression of human agrin (4-8) in Pichia pastoris

The 50kD active fragment (portion) of human agrin 4-8 was cloned by PCR using a primer containing a portion of the S. cerevisiae α mating factor pre-pro secretion signal and the 5' end of the region encoding the 50kD agrin fragment (GTATCTCTCGAGAAAAGAGAGGCTGAAGCT AGCCCCTGCCAGCCCAACC [SEQ ID NO:23], and a primer containing sequences from the region 3' of the agrin coding region and a NotI site (AATAGTGCGGCCGCCAACACTCAGGCAAGAAAATCATATC [SEQ ID NO:24]). After PCR the fragment was digested with XhoI, which recognizes sequences in the 5' primer, and NotI, and was cloned into pPIC9 (Invitrogen) digested with XhoI and NotI. The resulting clone was digested with NotI and partially digested with NcoI to remove most of the PCRed agrin sequences. This region was replaced by a NotI-NcoI fragment of agrin from pRG541. PCRed regions were sequenced and shown to be wild-type. This clone, pRG543 was digested with SalI and transformed into Pichia pastoris by electroporation. Transformants were selected for a His+ Mut+ phenotype. Induction of the AOX1 promoter driving the expression of hAgrin was done by growing the cells in buffered glycerol-complex medium containing 0.5% glycerol, pH=6.0, for 24 hrs until the glycerol was exhausted, at which point methanol was added to a final concentration of 0.5%. The culture was centrifuged and the supernatant was dialyzed against PBS. The concentration of hAgrin was approximately 10ug/ml and was determined to be active in phosphorylation of MuSK receptor.

MARKED-UP VERSION

A-15B (SEQ ID NO: 36)

As set forth in Figure 15, the amino acid sequence of the 50 kD active portion of human agrin 4,8 is 492 amino acids long. A preprotrypsin signal sequence (Stevenson et al., 1986. Nucleic Acids Res. 21: 8307-8330) precedes a FLAG tag sequence (Hopp etal. 1988. Bio/Technology 6: 1204-1210); together, they constitute the first 23 amino acids. Thus the agrin 4,8 sequence begins with amino acid 24. Truncated molecules were created, each of which contained the signal sequence and FLAG tag (23 amino acids) followed by the agrin 4,8 sequence to which N-terminal deletions had been made to create portions of agrin (designated delta 3 through 9) as follows:

delta 3: agrin sequence starts with amino acid #60: QTAS... (SEQ ID NO: 25)
delta 4: agrin sequence starts with amino acid #76: NGFS... (SEQ ID NO: 26)
delta 5: agrin sequence starts with amino acid #126: VSLA... (SEQ ID NO: 27)
delta 6: agrin sequence starts with amino acid #178: GPRV... (SEQ ID NO: 28)
delta 7: agrin sequence starts with amino acid #222: GFDG... (SEQ ID NO: 29)
delta 8: agrin sequence starts with amino acid #260: ASGH... (SEQ ID NO: 30)
delta 9: agrin sequence starts with amino acid #300: AGDV... (SEQ ID NO: 31)

All of the sequences continue to the terminal amino acids PCPTP, as with the 50kD agrin.

The truncated molecules were made as follows: PCR primers were designed consistent with the nucleotide sequences encoding the first and last ten amino acids of each construct. Included in the 5' primer was sequence data to append the preprotrypsin signal sequence and "FLAG-tag" to the amino terminus of each agrin fragment. Thus, the shortest truncated molecule (delta 9) contains the signal sequence and FLAG tag and the human agrin sequence from amino acid 300 to 492 of human c-agrin 4,8. DNA encoding the "delta" forms of truncated c-agrin 4,8 was then cloned into a eukaryotic expression vector, and transient transfections were performed as previously described (Glass, D., et al., 1991, Cell 66: 405-413; Ip, N.Y., et al., 1992, PNAS (USA) 89: 3060-3064).

9. A host-vector system for the production of a polypeptide having the biological activity of human agrin which comprises the vector of claim 8, in a suitable host cell.

10. The host-vector system of claim 9, wherein the suitable host cell is a bacterial cell, yeast cell, insect cell, or mammalian cell.

11. A method of producing a polypeptide having the biological activity of human agrin which comprises growing cells of the host-vector system of claim 9 or 10, under conditions permitting production of the polypeptide and recovering the polypeptide so produced.

12. A method of promoting the growth, differentiation or survival of a MuSK receptor expressing cell comprising administering to the cell an effective amount of agrin.

13. The method of claim 12, wherein the MuSK receptor expressing cell is a cell which is normally found in muscle, heart, spleen, ovary or retina.

14. The method of claim 12 or 13, wherein the MuSK receptor expressing cell is a cell which has been genetically engineered to express the MuSK receptor.

(AMENDED)
15. An antibody capable of specifically binding the ~~polypeptide of claim 5 or 6~~ the active portion of human agrin.

16. A monoclonal antibody of claim 15.

17. A polyclonal antibody of claim 15.

37. The method of claim 30, wherein the disease or disorder is an acquired (toxic or inflammatory) myopathy.

(Amended) encoding the active portion of human agrin,
38. A polypeptide ~~as defined in claim 5 or 6,~~ for use in a method of treatment of the human or animal body by therapy or in a method of diagnosis.

39. A polypeptide according to claim 38, for use in a method of treatment of the human or animal body of a disease or disorder that affects muscle.

40. A polypeptide according to claim 39, wherein the disease or disorder is muscle atrophy resulting from denervation due to nerve trauma, degenerative, metabolic or inflammatory neuropathy, peripheral neuropathy, or damage to nerves caused by environmental toxins or drugs.

41. A polypeptide according to claim 39, wherein the disease or disorder is muscle atrophy due to a motor neuronopathy.

42. A polypeptide according to claim 39, wherein the disease or disorder is muscle atrophy due to chronic disuse.

43. A polypeptide according to claim 39, wherein the disease or disorder is muscle atrophy due to metabolic stress or nutritional insufficiency.

44. A polypeptide according to claim 39, wherein the disease or disorder is muscle atrophy due to a muscular dystrophy syndrome.

45. A polypeptide according to claim 39, wherein the disease or disorder is muscle atrophy due to a congenital myopathy.

46. A polypeptide according to claim 39, wherein the disease or disorder is an acquired (toxic or inflammatory) myopathy.

(AMENDED)
47. Use of a polypeptide ~~as defined in claim 5 or 6~~ encoding The active portion of human agrin in the manufacture of a medicament for the treatment of a disease or disorder affecting muscle.

48. A pharmaceutical composition comprising a polypeptide as defined in claim 5 or 6 and a pharmaceutically acceptable carrier.

49. A diagnostic test kit for detecting the presence of human agrin in a sample, said kit comprising an antibody as defined in any of claims 15 to 17, and means for determining whether or not the antibody binds to human agrin, thereby allowing detection of the presence of human agrin in the sample.

(AMENDED)
50. A method of treating a patient suffering from a disease or disorder affecting muscle comprising administering to the patient an effective amount of the nucleic acid molecule comprising a nucleotide sequence encoding The active portion of human agrin, ~~of claim 1, 2, 3 or 4,~~ or a derivative thereof.

(AMENDED)
51. A nucleic acid molecule comprising a nucleotide sequence encoding The active portion of human agrin ~~as defined in claim 1, 2, 3 or 4,~~ or a derivative thereof, for use in a method of treatment of the human or animal body by therapy or in a method of diagnosis.

52. A nucleic acid molecule according to claim 51, for use in a method of treatment of the human or animal body of a disease or disorder that affects muscle.

MARKED-UP VERSION

53. (AMENDED) Use of a nucleic acid molecule comprising a nucleotide sequence encoding The active portion of human agrin, as ~~defined in claim 1, 2, 3 or 4~~, or a derivative thereof, in the manufacture of a medicament for the treatment of a disease or disorder affecting muscle.

54. (AMENDED) A ~~pharmaceutical~~ composition comprising a nucleic acid molecule comprising a nucleotide Sequence encoding The active portion of human agrin, as ~~defined in claim 1, 2, 3 or 4~~, or a derivative thereof, and a ~~pharmaceutically acceptable~~ carrier.

55. A nucleic acid molecule according to claim 1, 2, 3 or 4, or a derivative thereof, substantially as hereinbefore described.

56. (AMENDED) An expression ~~vector according to claim 7 or an expression vector according to claim 8~~, comprising a nucleic acid molecule comprising a nucleotide Sequence encoding The active portion of human agrin wherein The nucleic acid molecule is operatively Linked To an expression control sequence, substantially as hereinbefore described.

57. (AMENDED) A host vector system for The production of a polypeptide having The biological activity of human agrin which comprises The vector ~~according to claim 9, substantially as hereinbefore described~~ of claim 56 in a suitable host cell.

58. A method according to claim 11, 12, 18, 30 or 50 substantially as hereinbefore described.

59. An antibody according to claim 15 substantially as hereinbefore described.

60. A polypeptide according to claim 39 substantially as hereinbefore described.

61. Use according to claim 47 or 53 substantially as hereinbefore described.

62. A pharmaceutical composition according to claim 48 or 54 substantially as hereinbefore described.